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Tubulointerstitial responses in the progression of glomerular diseases: Albuminuria modulates $\alpha_v\beta_5$ integrin

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Tubulointerstitial responses in the progression of glomerular diseases: Albuminuria modulates $\alpha_v\beta_5$ integrin. Proteinuria represents one of the most unfavorable prognostic factors in the progression of nephropathies. Several lines of evidence support a role for proteinuria *per se* in the development of interstitial fibrosis, albeit the molecular mechanisms are still unknown. We investigated the potential role of integrins expressed on tubular cells in regulating the synthesis and organization of interstitial matrix or as mediators of tubulointerstitial damage in conditions mimicking the nephrotic milieu. Under basal conditions, cultured tubular cells highly expressed $\alpha_3\beta_1$ and, at focal contacts, $\alpha_v\beta_3$. In contrast, $\alpha_v\beta_5$ was weakly and diffusely distributed all over the plasma membrane. Cultures on a variety of matrix substrates (fibronectin, laminin, collagen types I and IV, vitronectin, von Willebrand factor, fibrinogen) did not induce any phenotypic change in integrin expression by tubular cells. Conversely, the addition of albumin resulted in a highly increased membrane expression of β_5, which was organized in typical focal contacts and was related to the dose of albumin added. Immunofluorescence, flow cytometry, immunoprecipitation and RT-PCR experiments argue for a complex mechanism that includes increased post-transcriptionally regulated protein synthesis, accelerated conversion of precursors to mature forms, and increased surface delivery to discrete adhesive structures. Up-regulation of the β_5 chain in tubular cells was confirmed in 9 out of 11 kidney biopsies from proteinuric glomerulonephritides including membranous and focal sclerosing glomerulonephritis, while it was not expressed in nonproteinuric kidneys including five biopsy specimens. This is the first report indicating that proteinuria up-regulates the surface expression and distribution of a specific integrin chain on tubular cells. These observations suggest the participation of integrins in a hitherto unexplored mechanism of tubulointerstitial responses to glomerular injury.

Clinical [1–6] and experimental [7–9] evidence suggests that the progressive functional failure occurring in chronic renal diseases mostly depends on tubulointerstitial damage. Events with different pathogenesis lead to similar histopathological lesions, that is, the constant presence of tubular atrophy, mononuclear cell infiltrate and interstitial sclerosis in the advanced phases of all nephropathies. This fact suggests that multiple stimuli converge upon the interstitium through common final mechanisms [reviewed in 10–13]. Of particular interest is the evolution of some types of glomerulonephritis toward tubular atrophy and interstitial sclerosis, occurring when the primary process involving the glomerulus has already resolved. Indeed, recent studies have shown that even the early phases of glomerular diseases are associated with tubular damage, as evidenced by the increased number of proliferating tubular cells [14]. In the last few years, interest on the mechanisms of chronic renal failure has shifted from glomerular to tubular cells [15], which can on the one hand be damaged by several mediators, and on the other trigger the release of potent factors that in turn lead to pathogenic interstitial phenomena.

A complex network of intercellular signals originating from glomerular cells, vascular endothelium, tubular and infiltrating inflammatory cells is envisioned to control the adaptation to renal injury. However, the biochemical basis of this communication network remains elusive. Tubular epithelial cells respond to different perturbations, including high glucose, TNF and LPS, cytokines, such as IL-1, MCP-1, IL-6, TNF-α, TGF-β (endowed with both chemoattractive and fibrogenic properties), and vasoactive substances such as angiotensin II, endothelin and nitric oxide [15, 16]. Upon activation by such stimuli, tubular cells may express molecules involved in the immune response such as MHC class I and II and ICAM-1 [17, 18]. Moreover, tubular cells regulate the synthesis and deposition of basement membrane and interstitial matrix components such as laminins, collagens and fibronectin. The contact between tubular cells and interstitial matrix is operated by specialized adhesion molecules, chiefly, integrins.

Integrins are α/β transmembrane heterodimers derived from multiple combinations of 15 α and 8 β chains [19, 20]. Integrins mediate adhesion of the basal surface of cells to the underlying substratum by recognizing extracellular matrix components and by interacting with different cytoskeletal molecules. At least during the adhesion processes *in vitro*, they cluster in specialized structures called focal contacts.

Integrin expression can be regulated, among other factors, by cytokines and antiadhesive proteins [21]. Moreover, some cytokines, including TGF-β, directly influence mRNA transcription of integrins [22], or modulate interstitial matrix synthesis and consequent rearrangement of integrins at focal contacts [23]. Accordingly, matrix proteins can modulate integrin expression and, by means of integrin-generated intracellular signals, regulate the

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Table 1. Monoclonal antibodies employed in this study

Clone	Specificity	Provider
MAR4	β_1	S. Ménard, Ist Nazionale Tumori, Milano, Italy
MAR6	α_6	S. Ménard, Ist Nazionale Tumori, Milano, Italy
F2	α_3	L. Zardi, Ist. Studio e Cura Tumori, Genova, Italy
IST4	fibronectin	L. Zardi, Ist. Studio e Cura Tumori, Genova, Italy
AA3	β_4	V. Quaranta, Scripps Research Inst., La Jolla, CA, USA
IA9	β_5	M. Hemler, Dana Farber Cancer Inst., Boston, MA, USA
CB43	Na^+/Ca^{++} pump	F. Malavasi, Università di Ancona, Ancona, Italy
Anti-VIII	factor VIII	F. Bussolino, Università di Torino, Torino, Italy
SAM-1	α_5	Immunotech, Marseille, France
Gi9	α_2	Immunotech, Marseille, France
VIN 11.5	vinculin	Sigma, St. Louis, MO, USA
Pancytokeratin	Pancytokeratin	Progen Biotechnik, Heidelberg, Germany
Pancytokeratin	Pancytokeratin	Immustain EURO/DPC, Liarberis, UK
Anti-albumin	human albumin	Dakopatts, Copenhagen, Denmark

synthesis, degradation, composition and organization of the matrix itself [24]. Taken together, these findings argue for a major role of integrins in the chain of events converging to the interstitium and responsible for the changes leading to sclerosis.

Tubular damage can derive from the actions of several mediators that are in turn generated by tubular cells in response to high grade proteinuria [25]. Both in clinical observations [26] and in experimental models [27–29], proteinuria represents one of the most unfavorable prognostic factors to sclerogenesis. Decreased proteinuria following pharmacological or dietetic therapy is associated with less tubulointerstitial impairment [30–33].

The aim of this study was to investigate the potential role of integrins in the transmission of tubulointerstitial messages, starting from the novel hypothesis that integrin expression in renal tubular cells can be modulated by molecules filtered during proteinuria. To this purpose we evaluated the *in vitro* responses elicited from tubular cells by several physiological and abnormal matrix components and by nephrotic range proteinuria. We also assessed the modifications of *in vivo* integrin expression during nephrotic syndrome by evaluating a set of human biopsy samples from either proteinuric or non-proteinuric patients.

Methods

Antibodies

The primary monoclonal antibodies (mAb) used in this study, and the investigators who provided them, are as follows (Table 1): MAR4, specific for β1, and MAR6, anti-α6 integrin, from S. Ménard (Istituto Nazionale Tumori, Milano, Italy); F2, anti-α3 integrin, and IST4, anti-fibronectin, from L. Zardi (Istituto per lo Studio e Cura dei Tumori, Genova, Italy); AA3, specific for β4 integrin, from V. Quaranta (Scripps Research Institute, La Jolla, CA, USA); IA9 antibody to β5 integrin from M. Hemler and R. Pasqualini (Dana Farber Cancer Institute, Boston, MA, USA); CB43 antibody to the Na^+/Ca^{2+} pump [34], from F. Malavasi (University of Ancona, Italy) and a mAb to coagulation factor VIII from F. Bussolino (University of Torino, Italy). MAb SAM-1 to α5 and Gi9 to α2 integrins were purchased from Immunotech (Marseille, France). MAb VIN11.5 to vinculin was from Sigma Chemical Co. (St. Louis, MO, USA). Anti-pan-cytokeratins Mabs were from Progen Biotechnik (Heidelberg, Germany) and Immustain EURO/DPC (Liarberis, UK). Anti-human albumin was from Dakopatts (Copenhagen, Denmark). For immunoperoxidase staining and immunofluorescence techniques, mAbs were used at a final Ig concentration of 10 to 40 μg/ml according to our previous experience; immunoprecipitations were performed with 4 μg mAb per sample. For control purposes, irrelevant antibodies were routinely used.

Cell cultures

Human tubular cells were isolated from cortical fragments obtained, with the patients' consent, from the normal part of kidneys surgically removed for renal carcinoma. The fragments were minced and passed through sieves of different pore size. The cell suspension obtained was washed and seeded on culture plates (Becton Dickinson, Lincoln Park, NJ, USA) containing RPMI 1640 medium (Sigma) supplemented with 20% fetal bovine serum (Sigma), 2 mM glutamine (Sigma), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

Cells were subcultured five times before use, and purity was assessed by positive staining with mAb CB43 to the Na^+/Ca^{2+} pump, negative staining for coagulation factor VIII and positive staining with a panel of anti-cytokeratin antibodies.

Immunofluorescence

After detachment with 0.05% trypsin-0.02% EDTA (Sigma), 5×10^4 cells were plated onto 1.4 cm^2 round glass coverslips in 24-well plates (Costar Corp., Cambridge, MA, USA). Cells were incubated for eight hours in complete medium. After removal of the medium and several washes in PBS, coverslip-attached cells were fixed for 10 minutes at room temperature in 3% formaldehyde (from paraformaldehyde) in PBS, pH 7.6, containing 2% sucrose. After rinsing in PBS, cells were permeabilized by soaking the coverslips for three to five minutes at 20°C in HEPES-Triton X-100 buffer (20 mM HEPES, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100).

Indirect immunofluorescence was performed as previously reported [35, 36]. Briefly, the primary Ab was layered onto fixed and permeabilized cells and incubated in a humid chamber for 30 minutes at 37°C. After rinsing in PBS-0.2% BSA, coverslips were incubated with the appropriate rhodamine-tagged secondary antibody (Dakopatts) for 30 minutes at 37°C in the presence of 2 μg/ml of fluorescein-labeled phalloidin (Sigma). Coverslips were mounted in Mowiol (Hoechst AG, Frankfurt/Main, Germany) and observed in a Zeiss Axiophot photomicroscope equipped with epifluorescence illumination and with planapochromatic oil immersion lenses. Fluorescence images were recorded on Kodak T-Max 400 film, exposed at 1000 ISO and developed in T-Max Developer for 10 minutes at 20°C. Five experiments were performed for each condition.

Indirect immunoperoxidase technique

Samples from 18 needle or surgical renal biopsies were snap-frozen in isopentane pre-cooled in liquid nitrogen, and embedded

in OCT 4583 (Miles Scientific, Naperville, IL, USA), and sectioned (4 to 6 μm) in a Reichert-Jung cryomicrotome. Uninvolved areas of tumor nephrectomy specimens were used as normal controls.

After air drying, cryostat sections were fixed in cold acetone and washed in PBS. Slides were then placed in a solution of H_2O_2 in methanol to quench endogenous peroxidase, washed in PBS and blocked with a PBS solution containing normal serum from rabbits (the host of the secondary antibody) and 0.01% avidin. Following another wash, sections were incubated in 0.005% biotin in PBS, washed again and then overlaid with 10 μg/ml primary antibody for thirty minutes in a moisture chamber at room temperature. Slides were rinsed in PBS, incubated for thirty minutes at room temperature with biotinylated rabbit anti-mouse IgG, rinsed again and incubated in avidin-biotin peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA). Finally, slides were developed for 5 to 10 minutes with a solution of 0.075% diaminobenzidine and 0.015% H_2O_2 in 0.05 M Tris. Sections were counterstained with Mayer's hematoxylin (Zymed, San Francisco, CA, USA) and mounted in Clearium (Surgipath, Canada) and examined with a Zeiss Axiophot photomicroscope equipped with 16× and 63× planapochromatic lenses. To control for specificity of the immunoperoxidase staining, parallel sections of some randomly selected biopsies were overlaid with murine monoclonal IgG antibody specific for Ewing's sarcoma cells (Signet Laboratories, Dedham, MA, USA) as a primary antibody, instead of the monoclonal anti-β5 integrin antibody.

Flow cytometry

Cells were detached in 1 mM EDTA, washed in PBS and incubated with the appropriate primary Ab at 4°C for 30 minutes. After two washes in PBS, cells were treated with fluorescein-labeled rabbit anti-mouse IgG (Dakopatts) for 30 minutes at 4°C, rinsed in PBS, and analyzed using an EPICS XL counter (Coulter, Hialeah, FL, USA) set to analyze 5,000 cells per sample. Data were elaborated by the Immuno-4 program (Coulter). Each antibody was assayed 4 to 6 times for each condition.

Metabolic labeling and extraction

A subconfluent tubular epithelial cell monolayer in a 75 cm^2 flask was washed twice with methionine- and cysteine-free medium (Sigma) supplemented with 5% dialyzed fetal bovine serum, glutamine and antibiotics, and cultured for three hours in the same medium. This medium was then removed and replaced by 4 ml of the same medium supplemented with 100 μCi/ml ^{35}S-Translabel (Amersham Corp., Arlington Heights, IL, USA). After an overnight incubation in the radioactive medium, cells were washed three times with ice-cold PBS and extracted in 1 ml of 0.15 M NaCl, 50 mM Tris-HCl, pH 8.5, containing 1% sodium deoxycholate, 1% Triton X-100, 2 mM PMSF (phenylmethylsulphonyl fluoride), leupeptin (50 μg/ml), pepstatin (5 mg/ml) and aprotinin (20 U/ml). Extracts were centrifuged for 30 minutes at 15,000 g at 4°C, and supernatants were used for immunoprecipitations.

Immunoprecipitation

Immunoprecipitation experiments were performed as previously described [37]. Briefly, radiolabeled lysates were precleared with protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) pre-coated with normal mouse serum (Sigma). Precleared lysates were incubated with various selected mAbs, and immunocomplexes were collected by protein A-Sepharose CL-4B coupled with rabbit anti-mouse IgG (Pierce, Rockford, IL, USA) used to maximize capture of mAb. After several washes with extraction buffer, the final pellet was boiled in Laemmli buffer [38] without β-mercaptoethanol. Proteins in SDS were separated on 8% polyacrylamide gels by electrophoresis. Resultant gels were fixed, incubated in scintillation fluid, dried, and exposed to Amersham Hyperfilm with intensifying screens at −80°C for three days. Protein size was estimated using a series of markers including myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), egg albumin (46 kDa), and carbonic anhydrase (30 kDa) that had been prelabeled by ^{14}C methylation (Amersham Co.). Five single experiments were performed.

Cell treatments: Attachment to substrates

Round coverslips were coated for eight hours at 37°C with the following substrates: fibronectin, laminin, collagens type I and IV, gelatin (all from Sigma); vitronectin (from pooled normal human sera); von Willebrand factor (provided by A. Federici, University of Milano, Italy) or fibrinogen (donated by F. Bussolino, University of Torino, Italy) all at a concentration of 10 μg/ml in PBS. Cells were then plated and allowed to adhere for 4 or 18 hours at 37°C. At the end of the incubation, cells were fixed and processed for immunofluorescence as described above.

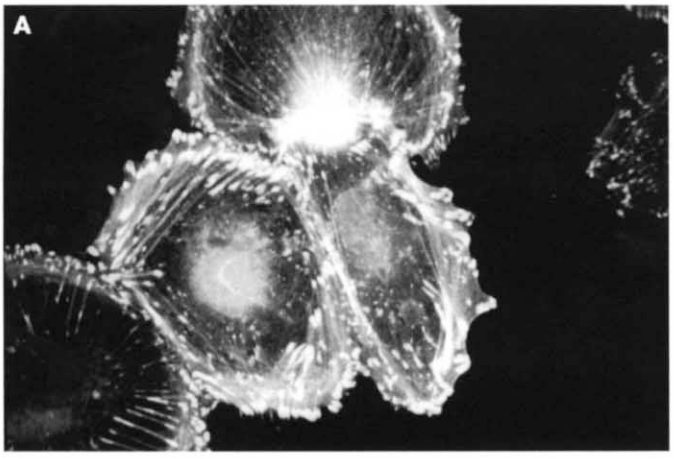
Incubation with albumin, IgG and lipopolysaccharide (LPS)

For immunofluorescence, 5×10^4 cells were allowed to adhere to round coverslips for eight hours at 37°C. Then the medium was substituted with complete medium with one of three additions: (a) human albumin (Human Albumin, Biagini, Pisa, Italy), starting from a final concentration of 35 mg/ml and going down to 10, 2, 1, 0.5, 0.2 mg/ml; (b) human albumin at 35 mg/ml plus human IgG (with intact Fc fragment) purified by alcoholic Cohn fractionation (Endobulin, Immuno, Pisa, Italy) at a final concentration of 15 mg/ml); or (c) LPS (Sigma) at a final concentration of 50 μg/ml. After a 5 or 20 hour incubation at 37°C in a humidified 5% CO_2 atmosphere, cells were fixed and immunofluorescence performed as described. To assess tubular epithelial cell function retention in culture, for the same conditions as above we performed a staining with the anti-human albumin antibody, to evaluate the *in vitro* uptake of albumin. For immunoprecipitation, one 75 cm^2 flask for each condition described above was prepared and incubated for five hours at 37°C in the CO_2 incubator. Metabolic labeling and immunoprecipitation were carried out as described at the appropriate time of incubation.

For flow cytometry analysis, one 75 cm^2 flask was used for each dilution of either human albumin or fatty acid-free bovine albumin (FAF-albumin, Sigma) starting from: (a) a final concentration of 35 mg/ml; (b) 2 mg/ml; (c) 1.5 mg/ml; (d) 1 mg/ml; (e) 0.5 mg/ml; down to (f) 0.02 mg/ml. Human IgG was added to each dilution to a final concentration of 15 mg/ml. Complete medium was used as a control. Flasks were incubated for five hours at 37°C. Cells were then detached, fixed, labeled with the antibodies and analyzed as described.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

After incubation in 1 mM EDTA for five minutes at 37°C, cells were recovered and resuspended in RNAzol (Cinna Biotecx



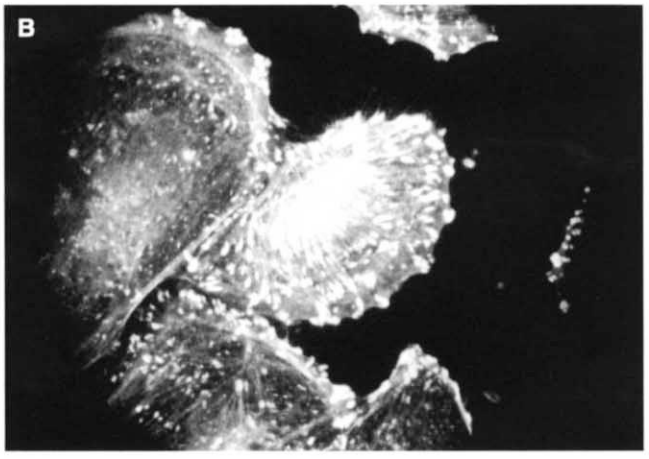


Fig. 1. *Immunofluorescence staining of human cultured tubular cells.* The cells were stained with mouse mAbs to α_3 (**A**) or β_3 (**B**) integrins followed by incubation with the appropriate rhodamine-tagged secondary antibody. Both integrin chains were found in streaks at the endings of microfilament bundles, strictly colocalizing with vinculin at structures corresponding to focal contacts (see Fig. 2). Original magnification, ×63. Figures are representative of 6 independent experiments, each with comparable results.

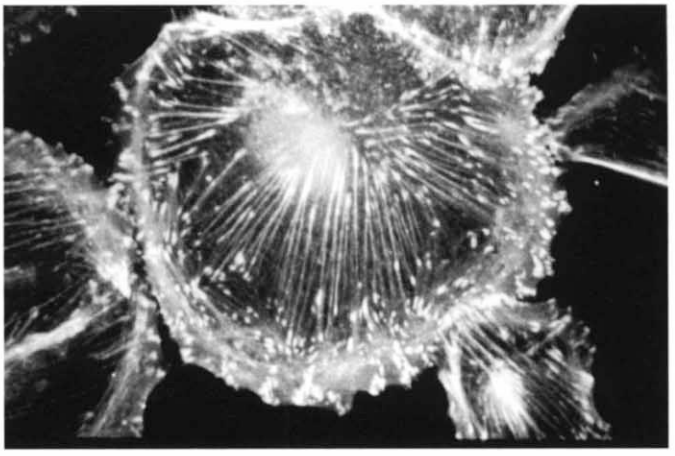


Fig. 2. *Immunofluorescence staining of vinculin-containing focal contacts in human cultured tubular cells.* A large number of vinculin-containing streaks were located at the endings of microfilament bundles at the substrate-attachment surface of cells. Original magnification, ×63 ($N = 6$ independent experiments).

Laboratories Inc., Houston, TX, USA) in a proportion of 0.2 ml for 10^6 cells. RNA was then extracted following the manufacturer's instructions. Briefly, after addition of 1/10 volume of chloroform, the suspension was vortexed and incubated at 4°C for 15 minutes. After centrifugation at 12,000 × g at 4°C for 20 minutes, the supernatant was extracted with an equal volume of isopropanol, incubated at −20°C for 45 minutes and re-centrifuged at 12,000 × g at 4°C for 20 minutes. The supernatant was then discarded and the pellet precipitated twice in 75% ethanol and centrifuged at 12,000 × g at 4°C for 15 minutes. The dried pellet was finally resuspended in distilled water and considered as total RNA. RNA yield and protein contamination were quantified by UV absorbance at 260 and 280 nm.

For RT-PCR, 1 μg of total RNA was reverse transcribed and then amplified by the polymerase chain reaction. Briefly, RNA was mixed with 2.5 mM random hexamers in 5 mM $MgCl_2$, 25 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1 mM of each dNTP, and 2.5 U/ml Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer-Cetus, Norwalk, CT, USA) in a final volume of 20 μl. Reverse transcription was carried out at 42°C for 15 minutes. This reaction was then amplified by bringing each sample to a final volume of 100 μl with 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 U *Taq* DNA polymerase (Perkin-Emler Cetus), and 0.15 M of the specific primer, in separate incubations. The following reaction temperatures were used for each cycle: denaturation for one minute at 95°C, annealing for 1.5 minutes at 55°C, and extension for 1.5 minutes at 72°C. Primers were: for the β5 integrin subunit:

forward 5′-CAGGATGGGGAGAACCAGAGC-3′
and reverse 5′-CTGGTCATCTTTCACGATGGT-3′;
for β-actin, used as control,
forward 5′-CCTTCCTGGGCATGGAGTCCTG-3′
and reverse 5′-GGAGCAATGATCTTGATCTTC-3′

The PCR reaction was performed semiquantitatively, that is, it was stopped at 5, 12, 18 and 25 cycles and a dilution series of the resultant DNA was performed before the final PCR amplification was run. This procedure avoided saturation and a plateau effect in the strongest signal. All the cDNA sample concentrations were furthermore normalized to the housekeeping gene β-actin. Aliquots of each reaction at different cycles were run on 2% agarose gels, visualized with ethidium bromide,under UV illumination, and the band intensities quantified by densitometry.

Results

Integrin expression in cultured renal tubular cells

To characterize the integrin expression pattern in primary cultures of renal tubular cells under normal conditions, we used a combined approach including immunocytochemistry, flow cytometry analysis and immunoprecipitation.

Cultured tubular cells expressed α_3 (Fig. 1A), β_1, α_v and β_3 chains (Fig. 1B). All these chains of integrins were abundantly distributed on the whole basal tubular cell surface along the endings of microfilament bundles together with vinculin (Fig. 2) and talin (not shown), indicating that these integrins are mainly organized in focal contacts. The β_5 chain was weakly and diffusely distributed all over the plasma membrane (see below). No appreciable staining for α_2, α_5 α_6 or β_4 could be observed.

Data were confirmed by flow cytometry analysis of suspended

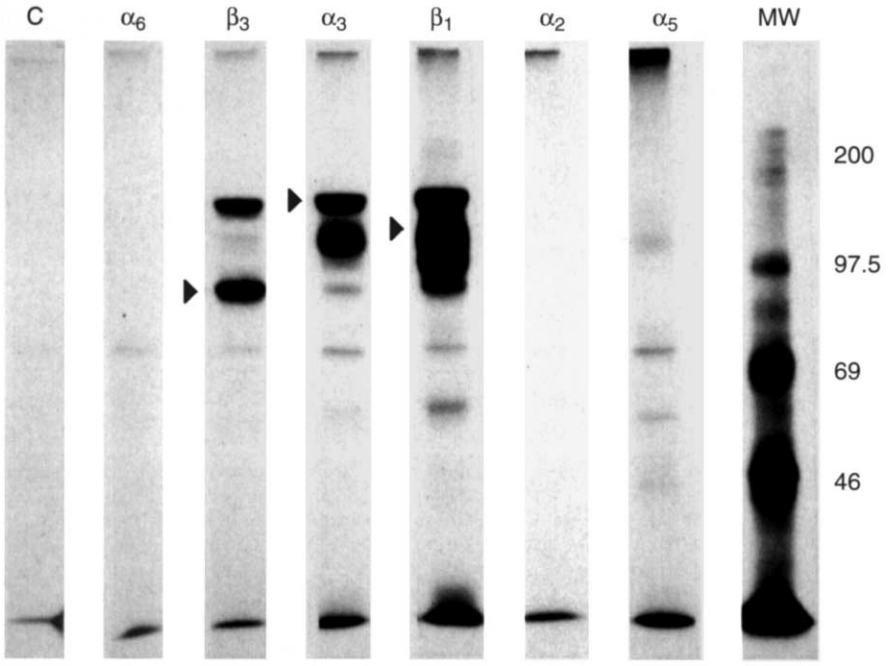


Fig. 3. *Immunoprecipitation of integrins.* Detergent lysates of metabolically labeled tubular cells were immunoprecipitated with control pre-immune mouse serum or the indicated mAbs as described in the **Methods** section. The eluates were analyzed by SDS-8% PAGE under non-reducing conditions. Either α_3 or β_1 specific mAbs immunoprecipitated multiple bands corresponding to α_3 and β_1 mature subunits (respectively 150 and 110 kDa) and precursors (140 and 95 kDa). A very faint 150 to 110 kDa doublet, corresponding respectively to α_5 and β_1, was brought down by anti-α_5 mAb. No chains were precipitated by anti-α_2 or α_6 mAbs. Anti-β_3 mAb yielded two broad bands representing α_v (150 kDa) and β_3 (95 kDa) (N = 4 independent experiments).

cells and by immunoprecipitation of radiolabeled lysates under non-reducing conditions. Both α_3 and β_1 antibodies immunoprecipitated multiple bands (Fig. 3) corresponding to α_3 and β_1 mature subunits (respectively 150 and 110 kDa) and their precursors (140 and 95 kDa). A very faint 150 to 110 kDa doublet was brought down by anti-α_5 antibodies. The absence of appreciable expression of α_5 by immunofluorescence methods and its detection by immunoprecipitation from metabolically labeled cells has already been observed in primary cultures of human epidermal keratinocytes, and is supposed to be due to lack of association of this heterodimer to definite adhesive structures [39]. α_2 and α_6 mAbs failed to precipitate any integrin subunit, confirming the immunofluorescence data.

The α_v integrin subunit was found to associate with both β_3 and β_5 subunits. Immunoprecipitation with an anti-β_3 mAb (Fig. 3) yielded two broad bands at 150 and 95 kDa, whereas in anti-β_5 immunoprecipitates (Fig. 4), three faint 150, 130 and 100 kDa bands were observed, the 130 kDa band presumably representing the α_v precursor [39]. Recovery of the α_v precursor was obtained following immunoprecipitation with a β_5-specific mAb and not with a β_3-specific mAb, possibly indicating the presence of a cytoplasmic pool of immature $\alpha_v\beta_5$ heterodimers preformed and stored in endoplasmic reticulum vesicles, but neither exposed at the cell surface nor organized in specialized adhesive structures [39].

Integrin expression in renal tubular cells cultured on different matrix substrates

To evaluate whether the presence of isolated extracellular matrix proteins could influence the integrin expression pattern or cell shape and adhesive properties, cells were plated onto selected substrates contained within the interstitium under physiological conditions (laminin, types I and IV collagen, fibronectin or vitronectin) or possibly present under pathological conditions such as inflammation or sclerosis (fibrinogen, denatured type I collagen, von Willebrand factor). Cells were then incubated for 4 or 18 hours, fixed and processed for immunofluorescence. The two time points selected were chosen according to time-course experiments, from 0 to 24 hours performed both on tubular cells and on other cell types, such as mesangial cells, keratinocytes and endothelium [22, 40, 41], demonstrating that integrin expression modifications were extremely rapid events, mostly happening within one hour and then remaining stable for a plateau. Under no circumstances did cells change their phenotype or integrin pattern in the two points considered, which were chosen to be able to catch early and eventual late events.

Integrin expression in renal tubular cells under conditions mimicking nephrotic syndrome. To evaluate the possible modulatory effect of molecules filtered to the pre-urine under conditions of altered glomerular permselectivity, we simulated a nephrotic proteinuria in the extreme case, assuming maximal derangement of the glomerular barrier, at an albumin concentration equal to that of plasma. Under these conditions, the most striking result was related to changes in the synthesis, surface expression, and cytoskeletal association of the $\alpha_v\beta_5$ integrin. As already mentioned, in renal tubular cells in basal conditions $\alpha_v\beta_5$ is poorly expressed and diffusely distributed on the basal plasma membrane, and is not organized in defined adhesive structures (Fig. 5A). In contrast, cells stimulated by albumin showed a clear $\alpha_v\beta_5$ staining that was organized in typical focal contacts (Fig. 5B). The same conversion to sharp focal positivity was obtained after incubation with albumin and IgG (Fig. 5C) and with the non-specific stimulatory agent LPS (Fig. 5D), while no modification was induced by using IgG alone (data not shown). LPS was included in the experiment as a positive control due to its capacity to induce strong cellular perturbations and activation in most cell populations binding to different cellular receptors [42] and acting

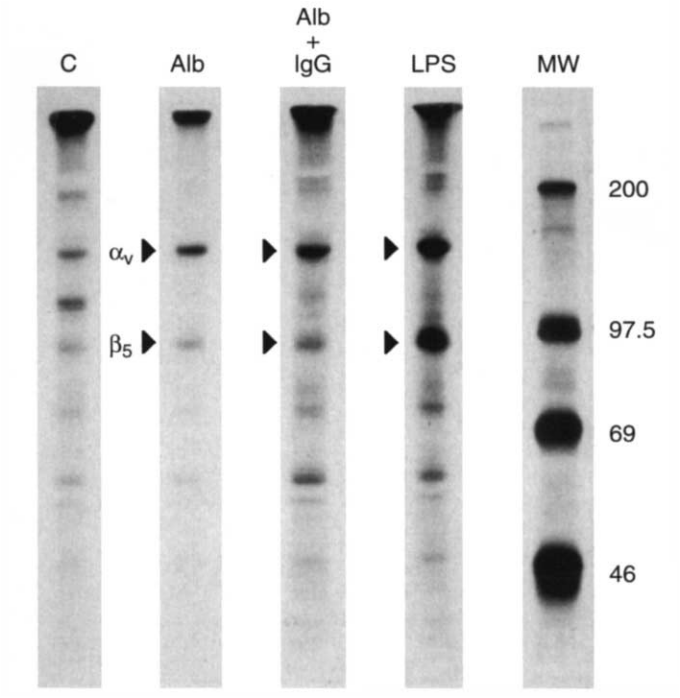


Fig. 4. *Immunoprecipitation of integrins from metabolically labeled detergent extracts.* Under basal conditions, anti-β_5 mAb immunoprecipitated three bands corresponding to β_5 (100 kDa), α_v (150 kDa) and presumably an α_v precursor (130 kDa). The band intensity increased in cells incubated in the presence of albumin (Alb) or co-stimulated by albumin and IgG (Alb + IgG), and peaked after LPS treatment. Little or no α_v precursor was precipitated in treated cells ($N = 3$ separate experiments).

through the activation of the NFκB factor [43]. LPS was previously demonstrated to induce several tubular events including the synthesis of TNF-α [44], the inducible form of nitric oxide synthase [45] and the interferon-inducible protein-10 [46].

The capacity to retain the cellular functions of *in situ* tubular cells was assessed by evaluating the cytoplasmic uptake of human albumin, which appeared to be conserved as displayed by the dense granular intracytoplasmic positivity by means of immunofluorescence (Fig. 6).

To investigate the synthesis and assembly of the receptor as well as its ratio of membrane exposure, we respectively immunoprecipitated metabolically labeled lysates and analyzed stimulated cells by flow cytometry. In anti-β_5 immunoprecipitates, two broad 150 and 100 kDa bands could be observed (Fig. 4). The band intensity increased in cells co-stimulated by albumin and IgGs and peaked after LPS treatment. No α_v precursor, precipitated from control cells, was precipitated in treated cells.

Similarly, flow cytometry analysis revealed that under basal conditions, 40 to 50% of the tubular cells expressed β_5 very faintly. In contrast, an increased surface positivity according to a linear dose-response curve was observed in treated cells over a range of concentrations of albumin, from the maximal 35 mg/ml to the 0.2 mg/ml level, similarly to the albumin concentration in the proximal tubule of rats with experimental puromycin nephrosis, and 0.02 mg/ml, the concentration of albumin physiologically present in the glomerular filtrate, from micropuncture data [47, 48]. The flow cytometry experiments were aimed at better analyzing the low concentrations of albumin, the effects of which were difficult to discriminate under the immunofluorescence microscope. These experiments showed that albumin induced an increase in β_5 expression starting from the 0.2 mg/ml concentration (64.3% of positive cells, with a mean fluorescence intensity of 1.74, compared to the 48.8% positive cells in basal conditions with 1.45 mean fluorescence intensity), at the 0.5 mg/ml albumin concentration (74.4% of positive cells, mean fluorescence intensity 1.81) and at 2.0 mg/ml (87.2% of positive cells, mean fluorescence intensity 2.1; Fig. 7).

The 35 mg/ml albumin concentration induced an even more intense effect in immunofluorescence, however, under flow-cytometry analysis albumin concentrations over 10 mg/ml were not able to induce distinguishable dose/response effects, as if the maximal activation was already obtained (data not shown).

Lipid-free bovine albumin failed to induce any modification of β_5 expression at each of the concentrations assayed. IgG alone did not cause any effect.

To elucidate the mechanisms regulating β_5 synthesis, we tried a semiquantitative RT-PCR approach on cells incubated under the same conditions used for flow cytometry analysis. In both untreated and treated cells, a specific 510 bp amplification product was present with band densities parallel to those of the housekeeper gene β-actin. Expresssion levels were almost identical in all samples. By stopping the reaction after different numbers of cycles, uniform levels of the transcript were achieved with kinetics analogous to the internal β-actin standard (Fig. 8). Thus, steady state mRNA levels do not appear to be affected by stimuli that increase immunoprecipitable protein levels.

Taken together, these data argue for a finely tuned regulation of $\alpha_v\beta_5$, possibly involving a post-transcriptionally-regulated increase of protein synthesis, accelerated conversion of precursor heterodimers to mature forms, and/or increased delivery to the cell surface, where the integrin clusters at discrete adhesive structures.

Integrin expression in renal sections in situ

Eleven of the patients we studied were subjected to renal biopsy to assess heavy proteinuria of presumed glomerular origin: four had IgM nephropathy, three had focal glomerulosclerosis, three had focal proliferative endocapillary glomerulonephritis without necrosis (1 each with post-infectious, systemic lupus or Henoch-Schönlein glomerulonephritis), and one proved to have membranous nephropathy upon renal biopsy. Among these patients, nine (82%) revealed strong immunoperoxidase staining for β_5 integrin chain on the surface of cells in some cortical tubules (Fig. 9A). On average, one third of the cortical tubular cross-sections exhibited the same quality (positive or negative) and intensity of staining. In contrast, five of the six patients without proteinuria in excess of 1.5 g/day had no β_5 integrin chain detectable in cortical tubules by immunoperoxidase (Fig. 9B). The one patient who did have moderate staining for β_5 integrin in 20% of his cortical tubular cross-sections was diagnosed with chronic tubular degeneration secondary to drug toxicicty, and in fact revealed extensive injury in the convoluted tubules. Staining for β_5 integrin was therefore significantly ($P < 0.01$) more frequent among patients with heavy glomerular proteinuria than in patients with non-glomerular renal disease and urinary proteinuria excretion below 1.5 g/day ($\chi^2 = 4.5$). This staining is considered specific, because no staining was

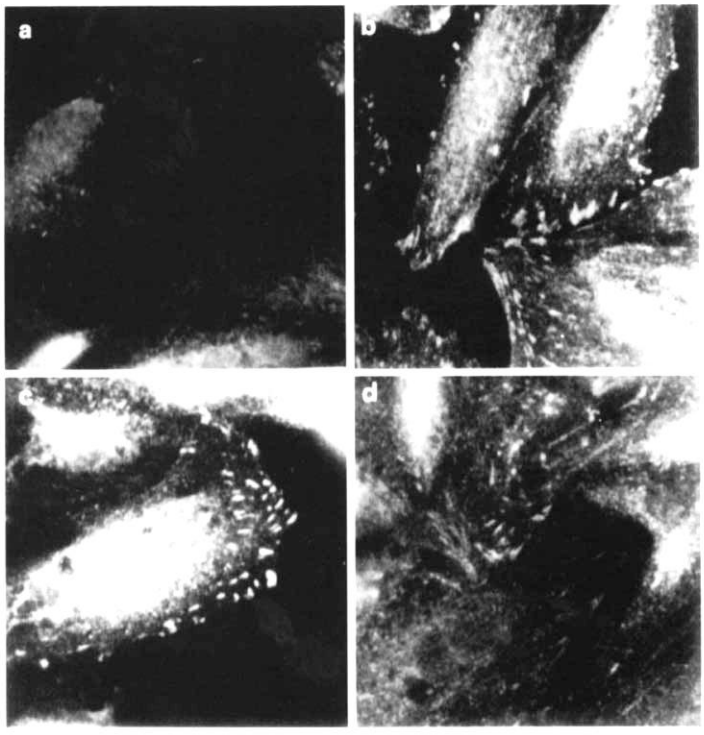


Fig. 5. *Immunofluorescence staining of the β_5 integrin chain in cultured human tubular cells.* Under basal conditions, $\alpha_v\beta_5$ was poorly expressed and diffusely distributed on the basal plasma membrane and was not organized in defined adhesive structures (**A**). In contrast, cells stimulated by albumin showed a clear $\alpha_v\beta_5$ staining, organized in typical focal contacts (**B**). The same conversion to sharp focal positivity was obtained after incubation with albumin and IgG (**C**) and with the non-specific stimulatory agent LPS (**D**).

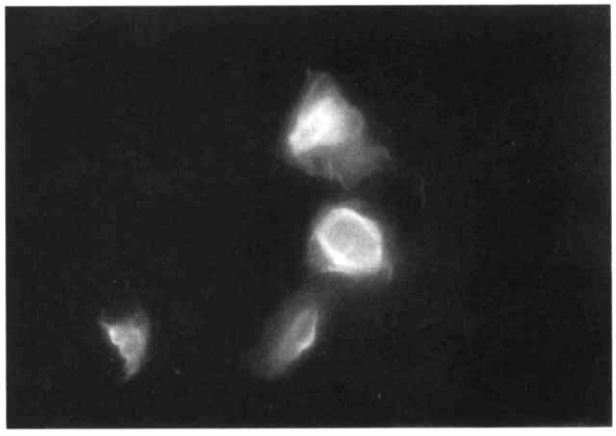


Fig. 6. *Immunofluorescence staining of human cytoplasmic albumin, expressing albumin uptake, after 4 hr incubation with human albumin (35 mg/ml final concentration), fixation, permeabilization and staining with an anti human albumin monoclonal antibody.*

observed when an irrelevenat primary antibody (specific for Ewing's sarcoma cells) was applied to parallel sections from four of the biopsies that stained positively for β_5 integrin.

The biopsy samples studied, even those displaying tubular β_5 integrin expression did not show any significant interstitial fibrosis, neither in the areas sorrounding the positive tubules nor in other compartments. This is possibly in accordance with the early onset of these nephropathies.

Moreover, no staining for β_5 integrin was present in any of the sections obtained from the normal parts of kidneys removed for carcinoma, which were the same kidneys used to establish the cell lines fore the *in vitro* experiments described above.

Discussion

Proteinuria apparently plays a key role in the development of interstitial fibrosis [25, 28–30]. Increased protein traffic through proximal tubular cells, activated to reabsorb and catabolize the filtered proteins, may give rise to a metabolic derangement of tubular epithelial cells, which in turn could affect the interstitium [49]. Tubular cells are active elements able to participate in phlogistic and immune processes by secreting cytokines, growth factors, vasoactive substances [reviewed in 15, 50], and molecules involved in antigen presentation and signal transduction [17, 51]. However, the specific mechanisms linking proteinuria to tubular responses are still unclear.

Several filtered proteins can exert specific effects. For instance, transferrin delivers iron ions to the intracellular acidic environment, where these ions catalyze the formation of reactive oxygen species [52], thus damaging the apical membrane of tubular cells and probably disturbing other cellular functions. Complement proteins, filtered during massive proteinuria, can be activated on the brush border of proximal tubular cells [53] with a consequent insertion of the membrane attack complex onto the tubular cell membrane [54], followed by profound cytoskeletal alterations, cytolysis [55], and possibly synthesis of cytokines and autacoids. Lipoproteins, filtered in the course of non-selective proteinuria, are specifically bound by membrane receptors and recycled within the cytoplasm, where they release the lipid moieties that can accumulate into lipid droplets, or be oxidized to toxic radicals [56, 57]. Moreover, it is conceivable that some of the responses evoked by LDL from mesangial cells, such as proliferation [58], expression of early nuclear genes and increased transcription of PDGF, fibronectin and other matrix proteins [59, 60], could also be elicited from tubular epithelial cells.

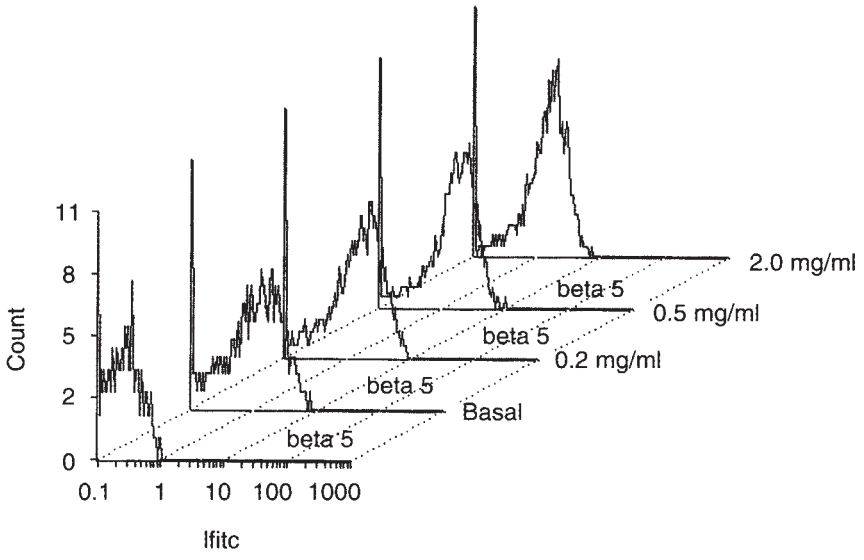


Fig. 7. *Flow cytometry analysis of β_5 chain expression on tubular cells incubated with human albumin The X axis shows fluorescence intensity; the Y axis shows relative cell number.* Human albumin induced a strong increase of β_5 expression, displayed by the right shift of the peak on the fluorescence intensity log scale and by the increase of the positive cells percentage, expressed by the hight of the peak.

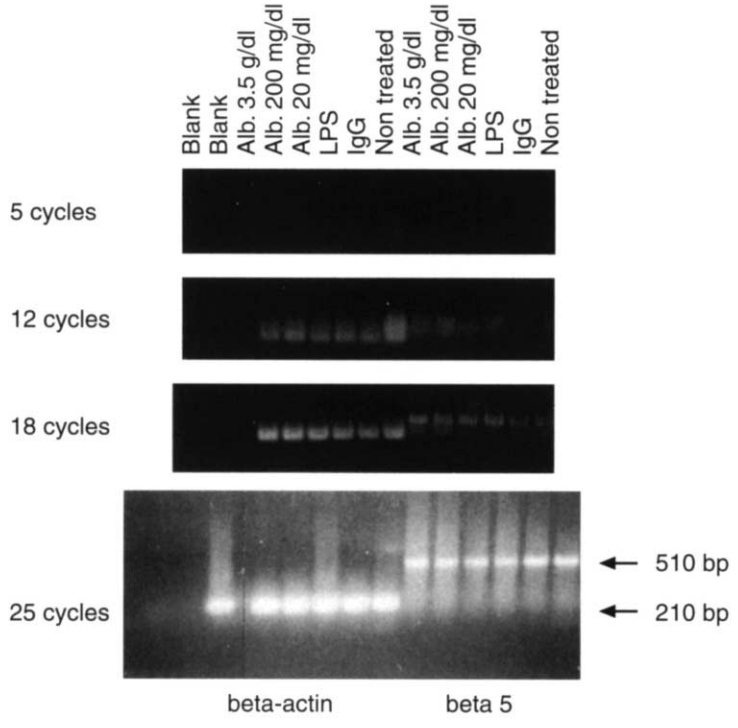


Fig. 8. *Semiquantitative RT-PCR analysis of the β_5 integrin transcript in cultured tubular cells under different conditions.* One microgram of the total RNA from untreated or treated cells was reverse transcribed and amplified with specific β_5 integrin oligonucleotides as well or with β-actin oligonucleotides as an internal control. The reaction was stopped at 5, 12, 18 and 25 cycles. In both untreated and treated cells, a specific 510 bp amplification product was present, with band densities parallel to those of the housekeeper gene β-actin. The amplificands were almost identical in all samples. For reactions stopped after different numbers of cycles, the transcript appeared to be wholly analogous to the internal β-actin standard.

Albumin physiologically carries different moieties of hydrophobic substances including hormones, vitamins, lipids. Upon albumin uptake by tubular epithelial cells, these substances could be released into the cytoplasm and be responsible for the effects attributed to albumin, with a mechanism analogous to that described for lipoproteins. It has been recently demonstrated [61] that tubular cells incubated with albumin charged with lipids release a lipid factor, endowed with macrophage chemotactic activity, that has a chromatographic pattern identical to a lipid factor extracted from urine of rats with experimental protein-overload proteinuria. In the case of massive proteinuria, therefore, the proximal tubule would metabolize large quantities of albumin and lipids, and produce a novel lipid that is able to attract macrophages to the interstitium. Lipid moieties carried by albumin have been shown to trigger many signal transduction pathways involving several effectors. One or more of these could modulate interactions between cells and the extracellular matrix, thus initiating a series of events potentially leading to monocyte/macrophage recruitment and interstitial fibrogenesis.

Integrins represent a critical interface between cells and their

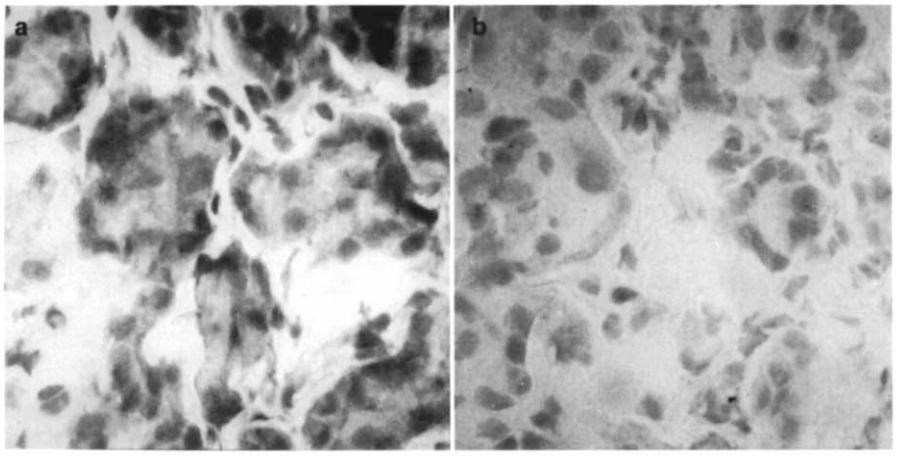


Fig. 9. *Immunoperoxidase staining of a nephrotic (***a***) and a normal (***b***) kidney section.* In the proteinuric condition, some cells, apparently in proximal convoluted tubules, strongly expressed the β_5 integrin chain (a). Conversely, tubular epithelium from normal kidney showed only a weak positivity for the β_5 subunit (b).

environment, including the extracellular matrix. In turn, integrins may play a major role in the synthesis, degradation and composition of the extracellular matrix, as well as in transmembrane signaling. Therefore, alterations in the expression and/or functional status of integrins may represent, in whole or in part, a metabolic connection between the contents of the tubular lumen and interstitial fibrosis. In this paper, we analyzed the expression of integrins by tubular cells in culture, and the modulation of integrin expression by proteins filtered when glomerular permselectivity is lost. Under basal conditions, cultured tubular cells mainly express $\alpha_3\beta_1$ and $\alpha_v\beta_3$, at the cytolemma enriched at focal contacts. The $\alpha_3\beta_1$ integrin is a promiscuous receptor that binds to laminin, fibronectin, collagen type I and nidogen/entactin, while $\alpha_v\beta_3$ may recognize a range of ligand sites on vitronectin, fibrinogen, von Willebrand factor, thrombin and thrombospondin. In contrast with reported data obtained from histological sections [62, 63], immunofluorescence as well as immunoprecipitation and flow cytometry analysis performed on cultured tubular cells could not detect any expression of α_6 chain. Conceivably, the loss of cell polarity due to the disruption of the tubular structure and the culture on plastic or glass induces changes in the panel of integrins expressed by tubular cells.

The basal integrin expression pattern was not modified by contact with physiological matrix components, such as laminin, type IV collagen (present in the tubular basement membrane), type I collagen (expressed in normal interstitium), fibronectin, or vitronectin, nor by contact with substrates potentially present in the matrix under pathological conditions, such as fibrinogen, von Willebrand factor, or denatured type I collagen. Accordingly, no changes in cell shape, cytoskeletal architecture or organization of adhesive structures could be observed. Taken together, these data suggest that, under our experimental conditions, modifications of the matrix environment are not a perturbing factor *per se*. Rather, any such changes might be an epiphenomenon of an early pathogenetic event arising from components acting directly on the tubular cell.

Consequently, we investigated the potential role of molecules abnormally present in the tubular lumen in cases of severe derangement of the glomerular ultrafiltration, that is, albumin and IgG. From this point of view, tubulointerstitial progression of glomerular disease would not depend on direct interstitial damage, but rather would rely upon noxious lumenal elements acting primarily on the tubule. Tubular cells might then respond to the abnormal lumenal composition by remodeling their adhesive apparatus, thereby exposing new provisional matrix receptors, thence triggering "inside-out" signals that might progressively convert the interstitium into a fibrotic tissue by activating fibroblasts and/or leukocytes.

The most striking modification of cultured tubular cells upon albumin treatment was the augmented synthesis, at the protein level, of $\alpha_v\beta_5$, as well as its surface exposure at focal contacts. This finding was validated by different techniques including immunofluorescence, flow cytometry, immunoprecipitation and RT-PCR analysis performed on cultured tubular cells, and further confirmed by the positive tubular staining with $\alpha_v\beta_5$ on renal sections from 9 out of 11 heavily proteinuric patients. To our knowledge, these results represent the first report of a selective alteration of the integrin-based adhesive machinery in tubular cells that have come into contact with concentrations of albumin similar to those found in states of massive proteinuria. The retargeting of preformed integrins to defined adhesive structures both in tissue sections and in primary cultures has already been observed for $\alpha_5\beta_1$ in psoriatic keratinocytes [22] and a comparable mechanism exists for platelets [40, 41] and for the fast activation of $\alpha_5\beta_1$ in resting CD4+ human T cells [64]. Moreover, a new α_v-based heterodimer ($\alpha_v\beta_6$) is specifically induced in conditions mimicking wound healing in keratinocytes [22]. However, the induction of α_v and the specific targeting of β_5 to focal contacts in addition to β_3, with resultant exposure of a new α_v-based heterodimer, cannot be explained simply. The only interpretation we can offer is that the signals derived from α_v in combination with distinct β chains are different, and imply complex phenotypic changes. The whole problem requires deeper investigation. Likewise, increased recovery of biosynthetically labeled $\alpha_v\beta_5$ integrin without altered steady state mRNA levels warrants added investigation. Specifically, the lipid content of albumin is implicated, since $\alpha_v\beta_5$ was recruited to focal contacts only by lipid-laden albumin, and not by albumin stripped of lipids.

The function and the regulatory mechanisms of the alternative vitronectin receptor $\alpha_v\beta_5$ are still under scrutiny, even though its biochemical and molecular features have been established long ago [65–67]. Ours, though, is the first finding of a molecule expressed on tubular cells that can be modulated by albumin, or

presumably by the lipid moieties carried by albumin. This modulated integrin possibly behaves as a mediator between the tubular lumen and the interstitium. Interestingly, this integrin can be expressed in a partially active form and be converted to a functionally active molecule by some still unidentified factors. Therefore, the $\alpha_v\beta_5$ heterodimer represents a potential convergence of different stimuli.

In summary, this study demonstrates that proteinuria can up-regulate the expression of a particular integrin that could represent a central mediator in a network of tubulointerstitial messages evoked by an increased protein load to tubular cells. This event, if protracted, might activate a cascade of events leading to tubular atrophy and interstitial fibrosis, and finally to progressive impairment of renal function.

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